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FOREWORD

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Christy Janett 7/28/00
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Introduction

Our lab identified an N-terminal sequence with significant homology to the adenomatous polyposis coli (APC) tumor suppressor gene, which is commonly associated with colon cancer. Possible functions of APC include the regulation of β -catenin protein degradation and signaling and microtubule mediated cell migration. β -catenin binds to the Tcf/LEF transcription factor complex and regulates the transcription of c-myc and cyclin D1, thus indicating that this pathway may be involved in cell cycle regulation. Truncating mutations in APC or mutations in certain N-terminal serine residues of β -catenin, result in increased β -catenin levels and increased transcriptional activation. Increased β -catenin levels have been found in multiple cancers, including breast and ovarian cancers, which have normal APC function. This suggests that there is another potential tumor suppressor gene involved in β -catenin regulation. During the course of the work reported here, the full-length sequence of a new APC-like gene, APCL/APC2 (originally called ALG19) was published. This project focuses on characterizing and determining the function of this new APC2 gene.

Body

We isolated a 1364 bp sequence from a human infant brain cDNA library by screening of the HGS/TIGR proprietary EST database. This partial sequence was 49% identical to human APC and contained an N-terminal dimerization domain. During the course of this work, a similar sequence was submitted to the public EST database. Shortly thereafter the full-length cDNA sequence was published as both APCL and APC2 (16;19). Overall hAPC2 is 35% identical to human APC and the protein product predicted to be slightly smaller than APC (245 kD vs 310 kD). However, the N-terminal region has much greater homology to APC than the C-terminal. The N-terminal dimerization domain has 68% identity to APC, the conserved domain 45%, the armadillo repeat region 76%, and the β -catenin binding region greater than 50%. The C-terminal region of APC is only 30% conserved in APC2.

Using a 1 kb sequence to the N-terminal region, four PAC clones (1K8, 17J21, 22K8, and 26K20) were isolated for APC2. FISH analysis using the four clones localized APC2 to chromosome 19p13.3. APC2 was then fine mapped by radiation hybrid mapping to the 800 kb region containing markers D19S883 and WI-19632. This particular region of 19p13.3 exhibits significant loss of heterozygosity (LOH) in many different cancers and is near the Peutz-Jeghers syndrome (PJS) associated gene, LKB1/STK11. PJS is characterized by intestinal hamartomas and increased risk of gastrointestinal, ovarian, pancreatic, and breast cancers (10). Even though there is significant LOH in this region, there are few mutations in the LKB1 gene in sporadic breast and colorectal cancers and adenoma malignum of PJS patients (1;3). In addition, although 50% of ovarian cancers contain an LOH on 19p13.3, LKB1 is not mutated indicating that another gene of significance in the development of cancer exists in this region (17). Therefore, APC2 could be a tumor suppressor gene affected by LOH of chromosome 19p13.3.

APC2 expression was determined by RT-PCR and Northern analysis of both cell lines and tissue. APC2 was expressed in a variety of cells and tissues, including breast, colon, brain, and ovary, at both the RNA and protein level. APC2 expression, like APC, is greatest in the brain; however, there are differing levels in different brain regions with very little expression in the cerebellum and cerebral cortex. Lymphoid tissues and lymphoma cell lines had no detectable APC2 at the mRNA or protein level with the exception of K-562 leukemia cells, which express low levels of APC2.

Both rabbit and chicken hAPC2 antibodies were affinity purified on an antigen coupled CNBr column. Western blot analysis determined that both antibodies were specific to hAPC2 with no cross-reactivity to APC. This was confirmed using the SW480 colon cancer cell line that contains a C-terminal truncated form of APC. Neither rabbit nor chicken hAPC2 antibodies detect this truncated APC protein even though it contains the conserved N-terminal. The largest form of APC2 is slightly smaller than APC in the HBL-100 and MDA-MB-468 breast cancer cell lines and corresponds to the predicted 245 kD molecular weight. To further determine specificity, we blocked the antibody with recombinant antigen before western blot analysis and found that all bands are specific to APC2. Western blot analysis showed that APC2 is expressed in many cells lines including SKBR3, SW480, MDCK, MDA-MB-157 and 436.

To investigate the localization of APC2 in the cell, we performed immunocytochemistry on several cell lines including SKBR3, MDCK, SW480, MDA-MB-157, and A549 lung carcinoma cells. MDCK cells are derived from the kidney of a normal canine and had been used in previous studies of APC (12). SKBR3 and MDA-MB-157 cells are breast cancer cell lines. Specific APC2 staining was similar in all cell lines and was observed diffusely in the cytoplasm as well as being associated with tubular structures adjacent to the nucleus that resembled the Golgi apparatus. Staining was also concentrated along filamentous structures and in what appeared to be lamellipodial membranes.

To confirm the localization of APC2 to the Golgi apparatus, cells were double-stained with anti-APC2 and PKC μ , a kinase known to associate with the Golgi (8). Co-localization of APC2 and PKC μ was observed for much of the Golgi stack indicating that APC2 is associated with certain regions of the Golgi where it co-localizes with PKC μ . APC2 staining is also associated with small vesicles/particles; however, staining with LysoTracker Red and the early endosomal marker antibody, EEA1, eliminated lysosomes and endosomes respectively.

A relationship between APC2 and actin filaments was observed in cells stained with phalloidin. However, not all actin filaments stained and APC2 appeared to be concentrated near actin-associated membrane ruffles and lamellipodia as well as cell-cell contact sites. Treatment with cytochalasin D, an actin disrupting agent, causes actin filaments to retract into clumps or balls mostly at the cell periphery but also throughout the cell. Following treatment with cytochalasin D, APC2 remained associated with the actin filaments in A549 cells and in MDA-MB-157 cells.

Endogenous APC is localized at the tips of microtubules in MDCK cells and is not associated with actin filaments (12). Consistent with this, cytochalasin D treatment did not affect APC staining but disruption of microtubules with nocodazole did (12). We confirmed that APC distribution was unaffected by cytochalasin D treatment. Similarly, APC2 did not co-localize with microtubules in cells double-stained for tubulin and APC2. In addition, APC and APC2 were not co-localized in the cell. Even though APC and APC2 can be found concentrated at the cell membrane and often in the same general area, co-localization is not detected upon double-staining. However, it should be noted that both APC2 and APC are present in the cytoplasm.

One function of APC is the regulation of β -catenin function and/or turnover. However endogenous wild-type β -catenin and APC do not co-localize or do so only transiently (12). We next wanted to investigate if β -catenin and APC2 co-localized in cells. Like APC2, some β -catenin staining

is associated with actin filaments. Upon cytochalasin D treatment, β -catenin staining is disrupted and remains associated with actin filaments in a pattern similar to that observed for APC2. To address if APC2, which also has β -catenin binding and regulation domains, was localized to β -catenin-associated structures we treated SKBR3 cells with 10^{-6} M RA for 24 hours. We showed previously that SKBR3 cells have very low levels of β -catenin protein (14). However, after treatment with RA β -catenin levels increase markedly and it becomes localized to the membrane and to cell-cell contact sites (4). After treatment of SKBR3 cells with RA, APC2 also localized to cell-cell contact sites and along the membrane in close association with β -catenin. However, APC2 has more of a punctate staining pattern than β -catenin.

SKBR3 cells treated with RA change morphology and actin filaments become concentrated along the membrane and cell-cell contact (5). APC2 co-localizes with actin filaments in both untreated and treated SKBR3 cells. After RA-treatment APC2 is found concentrated at the membrane at the leading edge of the cell adjacent to but not precisely co-localized with actin filaments. At cell-cell contact sites APC2 and actin filaments are more precisely co-localized. Untreated SKBR3 cells have very little, diffuse β -catenin staining, which is not associated with actin filaments; however, after RA treatment regions of co-localization between actin and β -catenin can be found. In untreated SKBR3 cells, only a few regions of co-localization can be found between APC2 and β -catenin as β -catenin levels are so low; however, after treatment APC2 and β -catenin association can clearly be seen at the membrane and especially at cell-cell contact sites. APC2 also co-localized with β -catenin in untreated MDCK and A549 cells. These cells, unlike SKBR3 cells, express β -catenin at the membrane in the absence of RA.

Cell fractionation shows that APC2 is primarily in the cytoplasmic fraction in both A549 and SKBR3 cells. Based on our immunocytochemistry, we hypothesized that after RA treatment APC2

would move to the membrane fraction of SKBR3 cells. Indeed, both APC2 and β -catenin move to the membrane fraction of RA treated SKBR3 cells.

Finally, we looked at the ability of APC2 to regulate β -catenin signaling. As shown previously, APC2 can inhibit β -catenin signaling in SW480 cells (11;16). Several studies have pointed to a role of PKC-like enzymes in the transmission of the wingless signal (6). We investigated the effects of Calphostin C, a diacylglycerol (DAG)-dependent protein kinase C (PKC) inhibitor, and bisindoylmaleimide (bis), which inhibits both DAG-dependent and independent PKC isoforms, on the ability of APC2 to inhibit β -catenin signaling. Calphostin C had little effect on APC2 inhibition of β -catenin signaling. This is consistent with our earlier work in which we showed that this inhibitor did not increase cytoplasmic β -catenin. (13). In contrast, bis almost completely reversed the APC2 mediated inhibition of β -catenin signaling. Bis also inhibits APC activity and increases cytoplasmic β -catenin (7;13). These results point to a role for atypical PKC-activity in the regulation of both APC and APC2 function.

GSK3 β forms a complex with axin, β -catenin, and APC, which can then regulate β -catenin turnover (9). Although the precise role of GSK3 β is not clear, Li⁺, which inhibits GSK3 β activity, leads to the accumulation of β -catenin in the cytoplasm (13;15). We recently showed that LiCl does not significantly inhibit the ability of APC to down-regulate β -catenin signaling (7). In the present study we found APC2 to be somewhat more sensitive to LiCl than APC. LiCl increases signaling 3 fold over APC2 alone compared to ~1.5 fold for APC. Another significant difference between APC and APC2 is the ability of APC2 to inhibit the signaling activity of a mutant S37A form of β -catenin that is resistant to inhibition by APC (7).

Key Research Accomplishments

- Identification of a new APC-like gene, APC2, which is overall 35% identical to APC.
- APC2 is located on chromosome 19p13.3 in a region containing markers D19S883 and WI-19632, a region of LOH predisposing to several different cancers including breast and ovarian.
- APC2 is expressed in most cells and tissues, including breast, colon, and ovary with greatest expression in the brain, which is similar to APC.
- APC2 is localized diffusely in the cytoplasm, is localized to the golgi apparatus, and is associated with actin filaments.
- Upon retinoic acid (RA) treatment, APC2 co-localizes with β -catenin and actin filaments at the membrane in SKBR3 breast cancer cells.
- APC2 regulates β -catenin signaling activity.

Reportable Outcomes

- Manuscript in preparation: "Chromosomal fine-mapping, localization, and regulation of human APC2"

Conclusions

It is important that the role of APC2 in actin associated events such as cell migration, and/or cell shape changes now be determined. APC and APC2 could cooperate in the cytoplasm or in association with microtubules and actin filaments respectively to control such processes as β -catenin signaling and cell motility as suggested by Barth et al (2). In addition interactions between microtubules and actin filaments occur during cell motility (18). The cellular location and many binding domains of APC2 suggest that it has multiple and perhaps dynamic functions. Finally, the chromosomal localization of APC2 to chromosome 19p13.3, a region of significant LOH, suggests that APC2 could be a tumor suppressor gene important in several different cancers, including breast cancer.

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**Chromosomal fine mapping, localization, and regulation
of human APC2**

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1 ABSTRACT

2 A second adenomatous polyposis coli (APC)-like gene, APC2 was recently described and
3 localized to chromosome 19. We have now fine mapped APC2 to a small region of chromosome
4 19p13.3 containing markers D19S883 and WI-19632, a region commonly lost in a variety of
5 cancers. APC2 is expressed in many different tissues and cell lines including brain, breast,
6 colon, and ovary. Endogenous APC2 is diffusely distributed in the cytoplasm and co-localizes
7 with both the Golgi apparatus and actin filaments. Unlike APC, APC2 and β -catenin remained
8 associated with actin filaments following treatment with the actin-disrupting agent, cytochalasin
9 D. In addition, APC2 co-localizes with β -catenin and actin filaments at the membrane of
10 SKBR3 cells upon retinoic acid treatment. Like APC, APC2 has the ability to down-regulate β -
11 catenin signaling and is sensitive to the PKC inhibitor bisindolylmaleimide. APC2 is more
12 sensitive than APC to inhibition of GSK3 with LiCl and, unlike APC, can inhibit the signaling
13 activity of a S37A mutant form of β -catenin. These results suggest that APC2 is involved in
14 actin associated events and could influence cell motility through interaction with actin filaments
15 as well as functioning independently or in cooperation with APC to down-regulate β -catenin
16 signaling.

17

18

1 INTRODUCTION

2 The APC tumor suppressor gene, located on chromosome 5q21, is associated with colon
3 cancer. Possible functions include the regulation of β -catenin protein degradation and signaling
4 and microtubule mediated cell migration (27;30;33). β -catenin binds to the Tcf/LEF
5 transcription factor complex and regulates the transcription of c-myc and cyclin D1, thus
6 indicating that this pathway may be involved in cell cycle regulation (17;22;32;37;43).
7 Truncating mutations in APC or mutations in certain N-terminal serine residues of β -catenin,
8 result in increased β -catenin levels and increased transcriptional activation (26;27;31;33).

9
10 APC is a large protein, approximately 320 kD, containing many different domains
11 including an N-terminal dimerization domain, a conserved domain of unknown function,
12 armadillo repeats, β -catenin binding and regulation domains, axin binding domains, a
13 microtubule binding domain, and a human discs large (HDLG) binding domain (33). A search
14 for APC-like genes in the HGS/TIGR (Human Genome Sciences, Inc., and The Institute for
15 Genomic Research) proprietary database of human expressed sequence tags (ESTs) resulted in
16 the identification of an N-terminal sequence with significant homology to the human, mouse,
17 frog, worm, and fly APC genes. During the course of our work, two studies published the full-
18 length sequence of this gene as APCL and APC2 (29;45). Like APC, APCL/APC2 interacts
19 with β -catenin and can decrease β -catenin levels and signaling activity in SW480 colon cancer
20 cells (29). A second APC-like gene has also been identified in *Drosophila* (12;24;51). We now
21 show that this gene, which we will call APC2, is located close to markers D19S883 and WI-
22 19632 on a region of chromosome 19p13.3 commonly lost in a variety of cancers (3;7;23).
23 APC2 is expressed in many different tissues and cell lines including brain, breast, colon, and

1 ovary. Importantly, APC2 is expressed in many of the same tissues and cell lines as APC,
2 indicating a non-redundant function. Antibodies against the N-terminal of human APC2 detect
3 endogenous APC2 associated with the Golgi apparatus and actin filaments, particularly those
4 filaments present at the leading edge of the cell and at cell-cell contact sites. Like APC, APC2
5 has the ability to inhibit β -catenin signaling and is sensitive to the PKC inhibitor
6 bisindolylmaleimide. APC2 is more sensitive than APC to inhibition of GSK3 with LiCl and,
7 unlike APC, can inhibit the signaling activity of a S37A mutant form of β -catenin.

MATERIALS AND METHODS

Identification and DNA sequencing of APC2: A homology search was performed using the human APC sequence against the HGS/TIGR (The Institute for Genomic Research and Human Genome Sciences, Rockville, MD) proprietary database of human expressed sequence tags (ESTs) (1;2). In 1997, an APC-like EST was identified from a human infant brain cDNA library, and the corresponding cDNA clone was recovered. DNA sequencing was carried out on both strands of the cDNA clone by using an automated ABI 373 DNA Analysis System (Applied Biosystems). RNA was isolated from SKBR3 cells by the RNazol method (Tel-Test, Inc.). The RNA served as template in RT-PCR reactions using sequence-specific primers and the Expand RT-PCR System (Boehringer-Mannheim), according to manufacturer's protocol. The amplified product was subcloned using a TA Cloning kit (Invitrogen). TA cloned RT-PCR products were sequenced as described above.

FISH Analysis and Fine Mapping: A 1kb cDNA fragment from the N-terminal region of APC2 corresponding to the recombinant protein used to make antibodies was used to screen a P-1 derived artificial chromosome (PAC) library (Human Genome FISH Mapping Resource Centre at the Ontario Cancer Institute). Four genomic PAC clones were identified: 1K8, 17J21, 22K8, and 26K20. Fluorescence in situ hybridization (FISH) to normal human lymphocyte chromosomes was used to map the genomic PAC clones to chromosome 19p13.3. Fine mapping was performed using radiation hybrid screening by PCR (Research Genetics, Inc.). Primer sequences (5'-GCTGCAGGAGCTGAAGATG; 5'-GTGGCTGGAGTTGTCCCTTA) were designed to yield a 120 bp product spanning the first exon/intron junction.

1 *Antibody Development:* A recombinant GST-fusion protein to the N-terminal region of APC2
2 (aa 1- 249) was produced in *E. coli* using the pGEX-4T-2 vector, isolated, and released by
3 protease cleavage (Pharmacia Biotech). This protein was used to inoculate both rabbit and
4 chicken (Rockland Inc., Gilbertsville, PA). Both the rabbit serum and IgY collected from the
5 chicken eggs was affinity purified on an antigen coupled CnBr column (XMMR website at
6 http://vize222.zo.utexas.edu/Marker_pages/methods_pages/affinity_col.html).

7
8 *Northern Analysis:* Human multiple tissue and human cancer cell line poly(A)+ RNA blots were
9 obtained from Clontech and processed according to the supplied manufacturer's protocol using a
10 probe to the N-terminal region of APC2.

11
12 *RT-PCR:* RNA was isolated by the RNazol method (Tel-Test, Inc.). RT-PCR was performed
13 using the Perkin-Elmer Gene Amp RNA PCR Core Kit. Primers to the N-terminal region (5'-
14 AGGAGCTAAGGGACAACCTCCA; 5'-TCCAGCAGCTCCTTGTC AAT) were designed to
15 yield a 600 bp fragment. These primers were shown to be specific to APC2 by sequencing of the
16 product by the above method as well as using wt-APC as a negative control.

17
18 *Western Blot:* Cells were grown to confluence in 150 mm dishes, washed twice with phosphate
19 buffered saline (PBS) and lysed for 10 minutes on ice in 1% HEPES lysis buffer containing 1%
20 Triton-X and protease inhibitors (1 mM sodium vanadate, 50 mM sodium fluoride, and
21 Boehringer Mannheim complete mini EDTA-free protease inhibitor cocktail tablet). Lysates
22 were centrifuged at 14,000 rpm at 4°C. Protein content was determined by the BCA protein
23 assay (Pierce). Cytoplasmic and detergent soluble and insoluble fractions were made as

described previously (31). Western blotting was performed as previously described using either rabbit or chicken APC2 antibody at 1 µg/ml, APC Ab-1 (Oncogene) at 1 µg/ml, or β-catenin (Transduction Laboratories) at 1:1000 (40). The blots were developed using chemiluminescent detection (Pierce). Specificity of the antibodies was determined by incubating recombinant APC2 antigen (10 µg/ml) with the antibody for 1 hour at room temperature before incubating the blot.

Immunocytochemistry: SKBR3, A549, MDA-MB-157, SW480, and MDCK cells were plated on 18 mm coverslips in 12 well plates at approximately 100,000 cells/well. In some experiments, SKBR3 cells were treated with 10^{-6} M retinoic acid (RA) for 24 hours. In other experiments, cells were treated with 2 µM cytochalasin D (Sigma) in media for 2 hours at 37°C. Antibody blocking with the immunogen was performed as described above. Both treated and untreated cells were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton. Purified chicken APC2 antibody was used at a concentration of 1 µg/ml and secondary antibody conjugated with fluorescein (Pierce) was used at 1:100 while secondary antibody conjugated with Texas Red (Rockland) was used at 1:150. Other primary antibodies and reagents were used at the following concentrations: normal IgY (Rockland) at 1 µg/ml, monoclonal β-catenin antibody (Transduction Laboratory) at 1:100 overnight at 4°C, polyclonal anti-APC (kindly provided by P. Polakis (27)) at 1:100 overnight at 4°C, monoclonal anti-tubulin (Sigma) at 1:2000, phalloidin (Molecular Probes, Inc) at 1:200 for 15 minutes, anti-PKCµ (Transduction Lab.) at 1:200, monoclonal anti-EEA1 (Transduction) at 1:1000, and LysoTracker Red (Molecular Probes, Inc.) at 0.1 µM in media for 3 hour incubation at 37°C (cells were not permeabilized when using LysoTracker Red). All primary antibodies were incubated for 1 hour

1 and all secondary antibodies were used at a 1:100 dilution for 1 hour at room temperature unless
2 otherwise noted above.

3
4 *LEF Reporter Assay:* SW480 cells were plated at ~100,000 cells/well in a 12 well plate. After
5 24 hours, the cells were transfected using the Lipofectamine Plus (GibcoBRL) method with
6 either a control PCDNA3-cat expression vector, APC2 full length cDNA (kindly provided by Y.
7 Nakamura), or wild-type APC (0.1 µg each) along with renilla (2 ng) and TopFlash (0.1 µg)
8 (44). Wild-type and S37A β-catenin (0.1 µg) were transfected with 0.3 µg of APC or APC2. All
9 transfections were done in triplicate and repeated at least three times with the LEF reporter
10 activity measured in lumens after 48 hours using the luciferase assay (Promega). Cells were
11 treated as indicated 12-16 hours after transfection and collected 36 hours later, with the
12 exception of LiCl which was added with fresh media 3 hours after transfection and collected 48
13 hours later. A dose response was also performed with each treatment and the optimal doses
14 chosen for these experiments.

1 RESULTS

3 Identification of a Novel APC-Like Gene. We isolated a 1364 bp sequence from a

4 human infant brain cDNA library by screening of the HGS/TIGR proprietary EST database.

5 This partial sequence was 49% identical to human APC and contained an N-terminal

6 dimerization domain. During the course of this work, a similar sequence was submitted to the

7 public EST database. Shortly thereafter the full-length cDNA sequence was published as both

8 APCL and APC2 (45;51). Figure 1A illustrates the domain structure of all known members of

9 the APC family. A number of conserved domains are variably present in APC family members.

10 The best studied of these, human APC has the following domains (33): 1. an N-terminal domain

11 that can mediate dimer formation between two APC monomers (19;42), 2. a conserved domain

12 of unknown function, 3. seven armadillo repeats, which in other proteins are thought to mediate

13 protein-protein interactions, 4. three 15 amino acid repeats, which can bind β -catenin

14 constitutively, 5. seven 20 amino acid repeats, which can bind and target β -catenin for

15 degradation: 6. three SAMP repeats, which can interact with axin (6), 7. a basic domain with

16 microtubule binding properties: 8. a discs large binding site at the extreme C-terminal. Mouse

17 and xenopus APCs have very similar structures (not shown). None of the other APC genes has a

18 disks large binding site. Overall hAPC2 is 35% identical to human APC and the protein product

19 predicted to be slightly smaller than APC (245 kD vs 310 kD). However, the N-terminal region

20 has much greater homology to APC than the C-terminal. The N-terminal dimerization domain

21 has 68% identity to APC, the conserved domain 45%, the armadillo repeat region 76%, and the

22 β -catenin binding region greater than 50% (Figure 1). The C-terminal region of APC is only

23 30% conserved in APC2. APC2 lacks the three 15-amino acid constitutive β -catenin binding

24 repeats and contains only five of the seven 20 amino acid repeats. The three axin binding SAMP

1 repeats in APC are poorly conserved in hAPC2. However, the SAMP repeats in mAPC2 can
2 bind axin/conductin (45).

3
4 APC2 is more closely related to human APC than are *Drosophila* APC (dAPC) and the *C.*
5 *elegans* APC related gene (ARG). dAPC does not contain an N-terminal dimerization domain or
6 microtubule binding domain, but does contain the conserved domain, the armadillo repeats, one
7 15-amino acid repeat, the 20-amino acid repeats, and the basic region (16). ARG, however, only
8 contains the armadillo repeats and two regions similar to the APC SAMP repeats (34). A second
9 *drosophila* APC gene, APC2/E-APC (19% identical to hAPC2), is more similar to the dAPC
10 gene (26% identical) in structure except that it is much smaller and is missing three of the 20
11 amino acid repeats as well as the basic domain (12;51;52). In addition, *Drosophila* APC2/E-
12 APC differs significantly from human and mouse APC2 in that it is missing the dimerization
13 domain and has retained two of the 15 amino acid constitutive binding β -catenin repeats.

14
15 **Chromosomal Localization and Fine Mapping.** Using a 1 kb sequence to the N-terminal
16 region, four PAC clones (1K8, 17J21, 22K8, and 26K20) were isolated for APC2. FISH analysis
17 using the four clones localized APC2 to chromosome 19p13.3, which confirms the previously
18 published chromosomal assignment (7,8). 19p13.3 is ~20 mb in size. The genomic sequence of
19 APC2 is ~40 kb and the coding sequence 7 kb. APC2 was then fine mapped by radiation hybrid
20 mapping to the 800 kb region containing markers D19S883 and WI-19632 using primers
21 designed to span the first exon/intron junction (Figure 1B). This particular region of 19p13.3
22 exhibits significant loss of heterozygosity (LOH) in many different cancers and is near the Peutz-
23 Jeghers syndrome (PJS) associated gene, LKB1/STK11. PJS is characterized by intestinal

hamartomas and increased risk of gastrointestinal, ovarian, pancreatic, and breast cancers (25). Even though there is significant LOH in this region, there are few mutations in the LKB1 gene in sporadic breast and colorectal cancers and adenoma malignum of PJS patients (3;7). In addition, although 50% of ovarian cancers contain LOH on 19p13.3, LKB1 is not mutated indicating that another gene of significance in the development of cancer exists in this region (46). Marker D19S216, which is 9.5 cM distal to marker D19S883, but not LKB1 itself, exhibits 100% LOH in sporadic adenoma malignum of the uterine cervix (23). Therefore, APC2 could be a tumor suppressor gene affected by LOH of chromosome 19p13.3.

APC2 Expression. APC2 expression was determined by RT-PCR and Northern analysis of both cell lines and tissue. APC2 was expressed in a variety of cells and tissues, including breast, colon, brain, and ovary, at both the RNA and protein level (Table I). APC2 expression, like APC, is greatest in the brain; however, there are differing levels in different brain regions with very little expression in the cerebellum and cerebral cortex (Figure 2A). Lymphoid tissues and lymphoma cell lines had no detectable APC2 at the mRNA or protein level with the exception of K-562 leukemia cells, which express low levels of APC2.

Both rabbit and chicken hAPC2 antibodies were affinity purified on an antigen coupled CNBr column. Western blot analysis determined that both antibodies were specific to hAPC2 with no cross-reactivity to APC (Figure 2B). This was confirmed using the SW480 colon cancer cell line that contains a C-terminal truncated form of APC. Neither rabbit nor chicken hAPC2 antibodies detect this truncated APC protein even though it contains the conserved N-terminal. The largest form of APC2 is slightly smaller than APC in the HBL-100 and MDA-MB-468

breast cancer cell lines and corresponds to the predicted 245 kD molecular weight (Figure 2B). To further determine specificity, we blocked the antibody with recombinant antigen before western blot analysis and found that all bands are specific to APC2 (not shown). Western blot analysis showed that APC2 is expressed in many cells lines including SKBR3, SW480, MDCK, MDA-MB-157 and 436 (Figure 2C). A characteristic pattern of immunoreactive species was observed. Three major bands larger than 200 kD and several smaller molecular weight species of ~121, 81, and 51 kD (not shown on this blot) were present consistently. Most cell lines express the three >200 kD species with varying levels of the smaller species (compare 121 kD band of SKBR3 with MDA-MB-436 and SW480). Other cell lines, for example MDA-MB-157, have significantly less of all bands except for two >200 kD species. The presence of multiple bands by western blotting with APC2 antibodies is similar to that observed with APC antibodies (36). In the case of APC, these bands most likely represent degradation products or some of the 16 known splice variants.

Sub-cellular Localization of APC2. To investigate the localization of APC2 in the cell, we performed immunocytochemistry on several cell lines including SKBR3, MDCK, SW480, MDA-MB-157, and A549 lung carcinoma cells. MDCK cells are derived from the kidney of a normal canine and had been used in previous studies of APC (30). SKBR3 and MDA-MB-157 cells are breast cancer cell lines. Although both rabbit and chicken antibodies exhibited a similar staining pattern by immunocytochemistry, the chicken antibody was exceptional and was used for these studies. Preimmune chicken IgY and antigen blocked antibody, as well as IgY prior to antigen affinity purification, were completely negative (Figure 3 A and B). Specific APC2 staining was similar in all cell lines and was observed diffusely in the cytoplasm as well as being

1 associated with tubular structures adjacent to the nucleus that resembled the Golgi apparatus
2 (Figure 3). Staining was also concentrated along filamentous structures and in what appeared to
3 be lamellipodial membranes.

4
5 To confirm the localization of APC2 to the Golgi apparatus, cells were double-stained
6 with anti-APC2 and PKC μ , a kinase known to associate with the Golgi (18). Co-localization of
7 APC2 and PKC μ was observed for much of the Golgi stack indicating that APC2 is associated
8 with certain regions of the Golgi where it co-localizes with PKC μ (Figure 4 A1-3). APC2
9 staining is also associated with small vesicles/particles; however, staining with LysoTracker Red
10 and the early endosomal marker antibody, EEA1, eliminated lysosomes and endosomes
11 respectively (results not shown).

12
13 **APC2 Association with Actin Filaments.** A relationship between APC2 and actin filaments
14 was observed in cells stained with phalloidin. (Figure 4 B1-3). However, not all actin filaments
15 stained and APC2 appeared to be concentrated near actin-associated membrane ruffles and
16 lamellipodia as well as cell-cell contact sites (also see Figure 3). This staining pattern was
17 similar for all but one of cell types tested. MDA-MB-157 cells had more pronounced actin-
18 associated APC2 staining throughout the cell and less at cell-cell contact sites. Treatment with
19 cytochalasin D, an actin disrupting agent, causes actin filaments to retract into clumps or balls
20 mostly at the cell periphery but also throughout the cell. Following treatment with cytochalasin
21 D, APC2 remained associated with the actin filaments in A549 cells (Figure 4 C1-3) and in
22 MDA-MB-157 cells (Figure 4 D1-3).

1 Endogenous APC is localized at the tips of microtubules in MDCK cells and is not
2 associated with actin filaments (30). However, overexpression of APC results in the decoration
3 of microtubules throughout the cell (28;38). Consistent with this, cytochalasin D treatment did
4 not affect APC staining but disruption of microtubules with nocodazole did (30). We confirmed
5 that APC distribution was unaffected by cytochalasin D treatment (not shown). Similarly,
6 APC2 did not co-localize with microtubules in cells double-stained for tubulin and APC2 (results
7 not shown). In addition, APC and APC2 were not co-localized in the cell. Even though APC
8 and APC2 can be found concentrated at the cell membrane and often in the same general area,
9 co-localization is not detected upon double-staining (results not shown). However, it should be
10 noted that both APC2 and APC are present in the cytoplasm.

11
12 **APC2 Association with and Regulation of β -catenin.** One function of APC is the regulation of
13 β -catenin function and/or turnover. However endogenous wild-type β -catenin and APC do not
14 co-localize or do so only transiently (30). If an N-terminal truncated stable form of β -catenin is
15 expressed in MDCK cells, then β -catenin can be found co-localized with APC (5). We next
16 wanted to investigate if β -catenin and APC2 co-localized in cells. Like APC2, some β -catenin
17 staining is associated with actin filaments (Figure 4 E1-3). Upon cytochalasin D treatment, β -
18 catenin staining is disrupted and remains associated with actin filaments in a pattern similar to
19 that observed for APC2 (Figure 4 F1-3). To address if APC2, which also has β -catenin binding
20 and regulation domains, was localized to β -catenin-associated structures we treated SKBR3 cells
21 with 10^{-6} M RA for 24 hours. We showed previously that SKBR3 cells have very low levels of
22 β -catenin protein (39). However, after treatment with RA β -catenin levels increase markedly
23 and it becomes localized to the membrane and to cell-cell contact sites (see Figure 5 C2 and D2;

(8)). After treatment of SKBR3 cells with RA, APC2 also localized to cell-cell contact sites and along the membrane in close association with β -catenin (compare Figure 5 A2 and B2). However, APC2 has more of a punctate staining pattern than β -catenin.

SKBR3 cells treated with RA change morphology and actin filaments become concentrated along the membrane and cell-cell contact sites (Figure 5 A1, B1, C1, and D1; (9)). APC2 co-localizes with actin filaments in both untreated and treated SKBR3 cells (Figure 5 A1-3 and B1-3). After RA-treatment APC2 is found concentrated at the membrane at the leading edge of the cell adjacent to but not precisely co-localized with actin filaments. At cell-cell contact sites APC2 and actin filaments are more precisely co-localized (Figure 5 B1-3). Untreated SKBR3 cells have very little, diffuse β -catenin staining, which is not associated with actin filaments (Figure 5 C1-3); however, after RA treatment regions of co-localization between actin and β -catenin can be found (Figure 5 D1-3). In untreated SKBR3 cells, only a few regions of co-localization can be found between APC2 and β -catenin as β -catenin levels are so low (see Figure 5 C2); however, after treatment APC2 and β -catenin association can clearly be seen at the membrane and especially at cell-cell contact sites (Figure 5 E1-3). APC2 also co-localized with β -catenin in untreated MDCK and A549 cells (results not shown). These cells, unlike SKBR3 cells, express β -catenin at the membrane in the absence of RA.

Cell fractionation shows that APC2 is primarily in the cytoplasmic fraction in both A549 and SKBR3 cells (Figure 6A and B). Based on our immunocytochemistry, we hypothesized that after RA treatment APC2 would move to the membrane fraction of SKBR3 cells. However, this is not the case (Figure 6B). To compare the amount of APC2 in the different fractions, equal cell

1 equivalents of each fraction instead of equal amounts of protein were loaded. APC2 remains
2 concentrated in the cytoplasmic fraction whereas β -catenin clearly moves to the membrane
3 fraction of RA treated SKBR3 cells. This result indicates that APC2 does not associate tightly
4 with the membrane or β -catenin at the membrane. However, it is likely that our extraction
5 conditions do not preserve a transient or low affinity interaction. That is, the change in
6 localization of APC2 noted by immunocytochemistry might not be detectable after cell-
7 fractionation. However, the RA treatment does alter the apparent molecular weight of the three
8 high molecular weight species of APC2 in the detergent soluble fraction but not in the
9 cytoplasmic fraction (Bands 1, 2, and 3). There is also significantly less of the splice
10 variant/degradation products at ~51 K (pair of bands denoted by the arrowhead) in the RA
11 treated cytoplasmic fraction compared to the untreated fraction.

12
13 Finally, we looked at the ability of APC2 to regulate β -catenin signaling. As shown
14 previously, APC2 can inhibit β -catenin signaling in SW480 cells (Figure 7; (29;45)). Several
15 studies have pointed to a role of PKC-like enzymes in the transmission of the wingless signal
16 (10). We investigated the effects of Calphostin C, a diacylglycerol (DAG)-dependent protein
17 kinase C (PKC) inhibitor, and bisindoylmaleimide (bis), which inhibits both DAG-dependent
18 and independent PKC isoforms, on the ability of APC2 to inhibit β -catenin signaling. Calphostin
19 C had little effect on APC2 inhibition of β -catenin signaling (Figure 7A). This is consistent with
20 our earlier work in which we showed that this inhibitor did not increase cytoplasmic β -catenin.
21 (31). In contrast, bis almost completely reversed the APC2 mediated inhibition of β -catenin
22 signaling (Figure 7A). Bis also inhibits APC activity and increases cytoplasmic β -catenin

(11;31). These results point to a role for atypical PKC-activity in the regulation of both APC and APC2 function.

GSK3 β forms a complex with axin, β -catenin, and APC, which can then regulate β -catenin turnover (20). Although the precise role of GSK3 β is not clear, Li⁺, which inhibits GSK3 β activity, leads to the accumulation of β -catenin in the cytoplasm (31;41). We recently showed that LiCl does not significantly inhibit the ability of APC to down-regulate β -catenin signaling (11). In the present study we found APC2 to be somewhat more sensitive to LiCl than APC (Figure 7B). LiCl increases signaling 3 fold over APC2 alone compared to ~1.5 fold for APC. Another significant difference between APC and APC2 is the ability of APC2 to inhibit the signaling activity of a mutant S37A form of β -catenin that is resistant to inhibition by APC (Figure 7B; (11)).

DISCUSSION

The APC-like gene we isolated is identical to the recently published APCL and APC2 sequences (29;45). However, our data show that this gene is not brain specific, as Nakagawa et al. reported, but is found in many different tissues including breast, ovary, brain, and colon as shown by Western blotting, RT-PCR, and Northern analysis. However, like APC there is considerably more APC2 in the brain than most other tissues.

Relationship of hAPC2 to other APC family members

APC2 is more closely related to the human APC gene than the Drosophila APC gene or C. elegans ARG gene. Human APC2 does not contain the three 15 amino acid repeats thought to constitutively bind β -catenin, or the HDLG binding sites, and, therefore, should not be able to constitutively bind β -catenin or bind the discs large protein. APC2 does contain five 20 amino acid repeats that, in APC, are involved in the down-regulation of β -catenin protein and signaling activity and is able to inhibit β -catenin/LEF/TCF reporter activity (35). The axin binding SAMP repeats of APC are poorly conserved in hAPC2. Mouse APC2 SAMP repeats are more similar to those found in APC (notably the presence of M in the SAMP repeat) and can bind axin (45). hAPC2 contains AAVP and SALP instead of SAMP. Because a mutated SAMP (AALP) cannot bind conductin it is possible that these sites in hAPC2 may not bind axin (6). The differences between human and mouse APC2 genes in this region indicate either that they have a different function or that axin binding is not required for human APC2 to function. It is also possible that

1 other axin/conductin-like genes exist which exhibit different specificities for the various APC
2 forms. A second drosophila APC gene does not contain the SAMP repeats but does contain two
3 constitutive β -catenin binding sites. dAPC2 is similar in structure to dAPC except it is missing
4 the C-terminal region. The relationship of hAPC2 to the drosophila APC2/E-APC is not clear.
5 dAPC2/E-APC is not closely related to hAPC2 in terms of sequence similarity. Taken together
6 with the existence of many, tissue specific splice variants of at least one APC gene, it is likely
7 that the various members of the APC family have multiple tissue and context-dependent
8 functions.

10 **Significance of the chromosomal location of APC2**

12 The chromosomal localization of APC2 to chromosome 19p13.3 is significant because
13 this region is associated with Peutz-Jeghers Syndrome (PJS) and exhibits significant loss of
14 heterozygosity (LOH) in several sporadic cancers. Patients with PJS are more susceptible to
15 breast, testis, gastrointestinal, and ovarian cancers (25). Loss of 19p13.3 occurs in many
16 sporadic cancers including those of the breast and is remarkably common in sporadic ovarian
17 carcinomas (~50%) (46). Ovarian cancers are also characterized by a high rate (~16%) of
18 stabilizing β -catenin mutations (48). However, mutations in the PJS gene, LKB1, are not present
19 in most of these sporadic cancers suggesting the existence of other tumor suppressor loci in this
20 region of chromosome 19 (7;46). Our fine-mapping analysis shows that APC2 is located in the
21 region of markers D19S883 and WI-19632 between the LKB1 gene and the site of 100% LOH
22 found in adenoma malignum of the uterine cervix (23). Therefore, like APC, APC2 could be a
23 tumor suppressor gene important in several cancers.

Subcellular localization of hAPC2

hAPC2 is diffusely distributed in the cytoplasm, is localized to the Golgi apparatus, and is associated with actin filaments. In some instances, such as lamellapodia or membrane ruffles, APC2 exhibits a punctate staining at the ends of actin filaments. Unlike APC, APC2 remains associated with the disrupted actin filaments following treatment with cytochalasin D. β -catenin also remains associated with actin filaments following this treatment, indicating a close association with APC2. APC2 co-localizes with β -catenin and actin filaments at the plasma membrane, and in well differentiated cells such as MDCK and RA-treated SKBR3 cells, at cell-cell contact sites. Recent studies show that E-APC/dAPC2 co-localizes with actin caps during *Drosophila* development and negatively regulates wingless signaling in the epidermis (14;24;52). These data suggest that even though sequence similarity is low, hAPC2 and dAPC2/E-APC may be functional homologues and that both may be involved in actin-associated events such as motility as well as in β -catenin signaling. In contrast, endogenous APC localizes near the ends of microtubules in a punctate pattern but does not associate with actin (30). Upon overexpression, APC associates with microtubules throughout the cell (28;38). It has been suggested that APC might be involved in microtubule regulated membrane protrusion and cell migration as well as inhibition of β -catenin signaling (4). The present study demonstrates that APC2 and APC are present in the same cells indicating that they are not precise functional homologues and have non-redundant roles. Although APC2 and APC do not co-localize at the membrane or cytoskeletal structures they are both present in the cytoplasm. Preliminary results show that they can exist in the same complex in this environment (results not shown). Taken together these findings suggest an intriguing scenario in which cytoplasmic APC and APC2

1 regulate related microtubule and actin-based functions and β -catenin signaling either
2 independently or in co-operation.

4 **APC2 regulation of β -catenin signaling**

6 Like APC, APC2 also can inhibit β -catenin signaling. However, this activity of APC and
7 APC2 is regulated somewhat differently. Both proteins are equally susceptible to inhibition of
8 atypical PKCs and both are equally resistant to inhibitors of DAG-dependent PKCs. Previous
9 studies show that the ability of APC to inhibit β -catenin signaling is relatively insensitive to
10 inhibition of GSK3 with Li^+ . In keeping with its role in wg/wnt signaling the present study
11 shows that APC2 activity, although not completely inhibited by Li^+ , is more sensitive than APC.
12 Most strikingly, a form of β -catenin (S37A), which is resistant to APC regulated ubiquitination
13 and degradation, is as sensitive as wild-type β -catenin to APC2 regulation. The role of GSK3 in
14 the regulation of β -catenin signaling activity is not clear. It was originally proposed that GSK3
15 directly phosphorylated a number of serine and threonine residues in the N-terminal of β -catenin
16 although this has never been shown directly by phosphoamino acid analysis (50). GSK3 can also
17 phosphorylate other members of the APC/axin complex and is now thought to mediate complex
18 assembly rather than or as well as directly phosphorylating β -catenin (15). As pointed out
19 previously the N-terminal stability regulating region of β -catenin contains a number of serine
20 and threonine residues, only two of which are adjacent to a proline residue and conform to a
21 modest GSK3 consensus (31). Importantly, serines 33 and 37 are present within a region
22 (DSGIHS) with significant similarity to sequences known to be targets for the I κ B kinase (IKK)
23 family (49). Phosphorylation of the analogous residues in I κ B by IKK leads to its association

1 with β TCRP which when complexed with skp and cullin targets the phosphorylated protein for
2 ubiquitination (21). Because β -catenin is targeted for ubiquitination by the same complex these
3 data suggest that, serines 33 and 37 in β -catenin, could also be phosphorylated by IKK, or a
4 related kinase (14). Other serine and threonine residues in the N-terminal could be targets for
5 GSK-3 or other kinases. Our demonstration that the S37A form of β -catenin is resistant to
6 inhibition by APC but not APC2 together with their differential sensitivity to Li^+ suggests that
7 the two APC forms prepare β -catenin for phosphorylation by different kinases. Either route may
8 be sufficient for the regulation of β -catenin signaling or both could be required.

9
10 It is important that the role of APC2 in actin associated events such as cell migration,
11 and/or cell shape changes now be determined. APC and APC2 could cooperate in the cytoplasm
12 or in association with microtubules and actin filaments respectively to control such processes as
13 β -catenin signaling and cell motility as suggested by Barth et al (4). In addition interactions
14 between microtubules and actin filaments occur during cell motility (47). The cellular location
15 and many binding domains of APC2 suggest that it has multiple and perhaps dynamic functions.

16
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Footnotes

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- 2
- 3 ¹ Abbreviations: APC, adenomatous polyposis coli; APC2, APC-like gene; ARG, *C. elegans*
- 4 APC related gene; bis, bisindoylmaleimide; DAG, diacylglycerol; dAPC, *Drosophila*-APC;
- 5 dAPC2, *Drosophila* APC2; EST, expressed sequence tag; hAPC2, human APC2; HDLG, human
- 6 discs large protein; HGS/TIGR, Human Genome Sciences and The Institute for Genomic
- 7 Research; mAPC2, mouse APC2; PAC, P-1 derived artificial chromosome; PJS, Peutz-Jeghers
- 8 syndrome; RA, retinoic acid

Figure Legends

Table I: Expression of APC2

APC2 is expressed in many different tissues and cell lines including brain, breast, colon, and ovary at both the mRNA and protein level as observed by northern blotting, RT-PCR, and western blotting.

Figure 1: APC2 alignment, chromosomal localization and fine mapping

A) APC2 is 35% identical to human APC overall; however, the N-terminal dimerization domain is 68% identical. APC2 contains the conserved domain, the armadillo repeats, and five of the 20 amino acid repeats necessary for β -catenin binding and down regulation. APC2 lacks the three constitutive β -catenin binding sites and the DLG binding site. The SAMP repeats, necessary for axin binding, are poorly conserved in hAPC2. B) APC2 was mapped to chromosome 19p13.3 by FISH analysis using PAC clones identified through screening with a 1 kb cDNA fragment from the N-terminal region of APC2. Fine mapping using radiation hybrid screening by PCR located APC2 to the region on chromosome 19p13.3 containing markers WI-19632 and D19S883.

Figure 2: Expression of APC2 and western blot of cell lysates comparing APC2 and APC.

A.) Northern analysis of human multiple tissue and human cancer cell line poly(A)+RNA blots using a probe to the N-terminal region of APC2. APC2 expression is highest in the brain with varying levels in different regions. B.) SW480 and HBL-100 cell lysates were used to compare affinity purified APC2 rabbit antibody (5 μ g/ml) to APC antibody-1 (1 μ g/ml). No cross reactivity could be found. The truncated form of APC, T-APC, can be seen in SW480 cells and

full length APC, FL-APC, can be found in HBL-100 cells. APC2 is slightly smaller than FL-APC with possible splice variants or degradation products visible as well. Although both antibodies detected several immunoreactive species these did not coincide. MDA-MB-468 cell lysates were used to characterize the affinity purified IgY chicken antibody. C.) Varying protein patterns of APC2 are observed by western blot analysis of several different cell lysates using the IgY antibody (1 μ g/ml). Equal amounts of protein (60 μ g) were loaded in each lane.

Figure 3: Immunocytochemical staining for APC2.

A.) SKBR3 cells were stained using preimmune IgY (3 μ g/ml). Little staining could be seen even in this overexposed image. B.) MDA-MB-157 cells stained using IgY antibody blocked with APC2 protein. C.) MDA-MB-157 cells stained for APC2 using APC2 IgY antibody (1 μ g/ml). APC2 can be seen concentrated along filamentous structures as well as concentrated along the membrane (arrows). D.) A549 cells were stained with APC2 affinity purified IgY antibody (1 μ g/ml). The arrow indicates staining resembling the Golgi apparatus surrounding the nucleus. E.) A549 cells stained for APC2 as above. The box indicates a region of small vesicles/particles concentrated in a lamellipodial membrane. F.) A549 cells again stained for APC2. The arrows indicate staining resembling the Golgi apparatus surrounding the nucleus and staining along actin filaments.

Figure 4: APC2 localization at the Golgi apparatus and actin filaments.

A549 cells double stained with APC2 and PKC μ or phalloidin. Cells were treated with 2 μ M cytochalasin D for 2hr. A1.) PKC μ clearly stains the Golgi apparatus. A2.) APC2 localizes to the Golgi apparatus. A3.) Double staining shows APC2 co-localization with PKC μ at the Golgi

apparatus. B1.) Phalloidin staining of actin filaments in A549 cells. B2.) APC2 is diffusely stained in the cytoplasm and appears to associate with actin filaments. B3.) Double staining shows APC2 associated with actin filaments. C1.) A549 cells treated with cytochalasin D and stained with phalloidin. Actin filaments are disrupted. C2.) Cells stained for APC2. APC2 staining in disrupted. C3.) Double staining shows that APC2 remains associated with actin filaments following treatment with cytochalasin D. D1-D3.) MDA-MB-157 cells treated with cytochalasin D and stained for actin and APC2 E1.) A549 cells stained for actin. The arrow indicates staining concentrated at cell-cell contact sites. E2.) Cells stained for β -catenin (1:100). The arrow indicates β -catenin in the same region as actin filaments. E3.) Double staining shows actin and β -catenin localized in the same region but rarely co-localized exactly. F1.) A549 cells treated with cytochalasin D and stained with phalloidin. F2.) Cells stained for β -catenin. The arrow indicates that β -catenin is disrupted similar to both actin and APC2. F3.) Double-staining shows that β -catenin remains associated with actin filaments.

Figure 5: APC2 association with actin filaments and β -catenin.

SKBR3 cells, both untreated and treated with 10^{-6} M RA, stained with either APC2, phalloidin, or β -catenin. A1.) Actin staining in untreated SKBR3 cells. The arrow indicates actin bundling at the membrane. A2.) Cells stained for APC2. The arrow indicates APC2 concentrated in the same region as actin. A3.) Double staining shows APC2 co-localization with actin filaments. In A1-A3 the microscope was focused on the cell surface, actin-containing structures. B1.) SKBR3 cells treated with RA and stained for actin. The actin filaments (arrow) are more organized in the treated cells. SKBR3 cells treated with RA are larger and more flattened. B2.) Cells stained for APC2. APC2 becomes much more concentrated along the membrane (arrow) and cell-cell

contact sites. B3.) Double staining shows APC2 at the edge of the cell with actin behind it. APC2 and actin are more co-localized at regions of cell-cell contact. C1.) SKBR3 cells stained for actin. C2.) Cells stained for β -catenin. SKBR3 cells have very little β -catenin and the staining is very diffuse. C3.) β -catenin and actin interaction is not apparent upon double staining. D1.) SKBR3 cells treated with RA and stained for actin. Again a more organized actin structure can be seen as indicated by the arrow. D2.) Upon treatment with RA, β -catenin becomes concentrated along the membrane and cell-cell contact sites as indicated by the arrow. D3.) Double staining indicates that after RA treatment, β -catenin and actin filaments co-localize along the membrane at cell-cell contact sites (indicated with the arrow). E1.) Distinct β -catenin staining along the membrane can be seen upon treatment with RA in SKBR3 cells (indicated with the arrowhead). F2.) APC2 staining also becomes more concentrated along the membrane after RA treatment (arrowhead). F3.) Double staining demonstrates that β -catenin and APC2 co-localize in regions along the membrane (arrowhead).

Figure 6: APC2 remains in the cytoplasmic fraction after RA treatment of SKBR3 cells.

A.) A549 cells were fractionated into cytoplasmic, detergent soluble and insoluble fractions. APC2 was predominantly found in the cytoplasmic fraction. To determine the relative amount of APC2 in each fraction, equal cell equivalents of each fraction (i.e. 1/10 of each sample) were loaded in these experiments instead of equal amounts of protein. This blot was stripped and reprobed for β -catenin, which is found primarily in the detergent soluble membrane fraction. B.) APC2 is located in the cytoplasmic fraction of both RA treated and untreated SKBR3 cells. However, β -catenin levels not only increase but translocate to the detergent soluble membrane fraction after RA treatment. Untreated SKBR3 cells have very little β -catenin protein. Also note

1 that decreased mobility of three >200kD APC2 species in the detergent soluble phase following
2 RA-treatment. In addition, RA-treatment decreases the intensity of two bands at ~51 K
3 (arrowhead) in the cytoplasmic fraction.

4
5 **Figure 7: APC2 regulation of β -catenin signaling.**

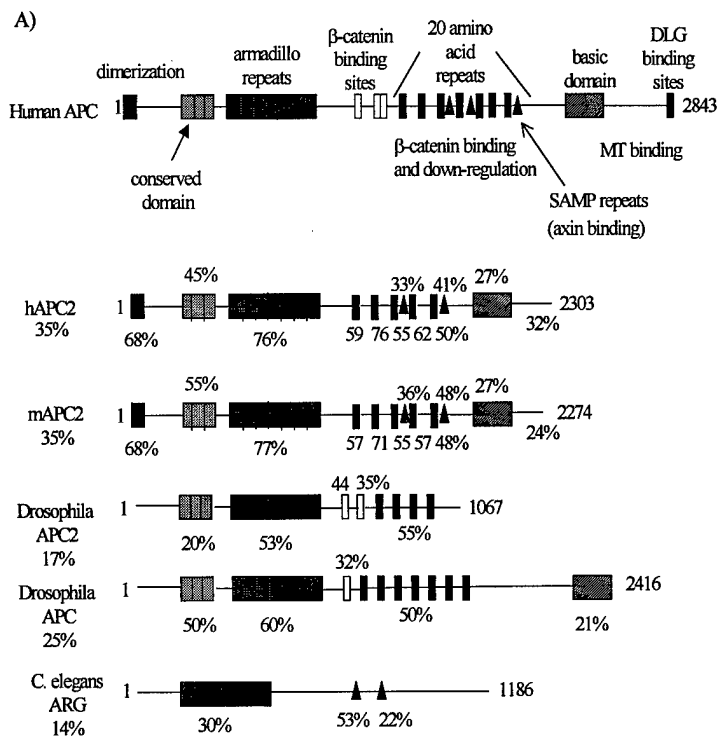
6 APC2 inhibits β -catenin signaling in SW480 colon cancer cells. A) The ability of APC2 to
7 inhibit β -catenin-regulated TOPflash activity is significantly reduced by bisindolmaleimide
8 (bis) but not by Calphostin C. In several experiments the effects of calphostin C ranged from 0
9 to 30% as shown here. Bisindolmaleimide effects ranged from complete reversal of APC2
10 activity to 80% reversal as shown here. All transfections were done in triplicate and data plotted
11 as percent inhibition. B) As shown previously, TOPflash activity induced by the S37A form of
12 β -catenin is resistant to APC inhibition (11). Li^+ reduced the ability of APC to inhibit TOPflash
13 activity in SW480 cells by up to 35% (as shown here). In other experiments Li^+ was completely
14 ineffective in inhibiting APC activity as shown previously (11). C) APC2 can inhibit TOPflash
15 signaling induced by S37A β -catenin as well as wild-type β -catenin. Li^+ consistently reduced
16 the ability of APC2 to inhibit TOPflash activity by 50-75%.

Table I: APC2 Expression

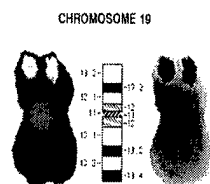
Cell Line	Type	APC2 Expression	Cell Line	Type	APC2 Expression	Tissue	APC2 Expression
A1N4	Breast	+ ²	SW480	Colon	+ ^{1,2}	Colon	+ ²
BT20	Breast	+ ¹	LS-123	Colon	+ ¹	Ovary	+ ²
HBL-100	Breast	+ ^{1,2}	T84	Colon	+ ¹	Testis	+ ²
MCF-7	Breast	+ ²	A549	Lung	+ ^{1,2}	Spleen	- ²
MCF-10A	Breast	+ ²	Calu-3	Lung	+ ¹	Periph. Bl. Luek.	- ²
MDA-MB-134	Breast	+ ²	G361	Melanoma	+ ²	Brain	+ ²
MDA-MB-453	Breast	+ ^{1,2}	SKOV3	Ovary	+ ¹	Sm. Intestine	+ ²
MDA-MB-231	Breast	+ ²	U87	Brain	+ ¹	Prostate	+ ²
MDA-MB-468	Breast	+ ^{1,2}	A-431	Epidermoid	+ ²	Thymus	- ²
MDA-MB-435	Breast	+ ¹	MOLT-4	Leukemia	- ²		
SKBR3	Breast	+ ^{1,2}	Burkitt's Raji	Lymphoma	- ²		
T47D	Breast	+ ^{1,2}	HL-60	Leukemia	- ¹		
ZR-75-1	Breast	+ ¹	K-562	Leukemia	+ ¹		

1 = protein, 2 = RNA

Figure 1



B)



Chromosome 19p13.3

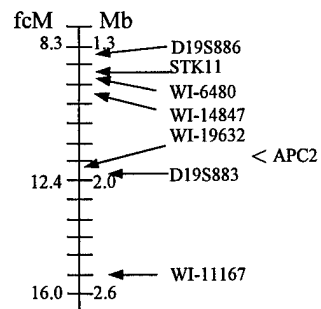


Figure 2

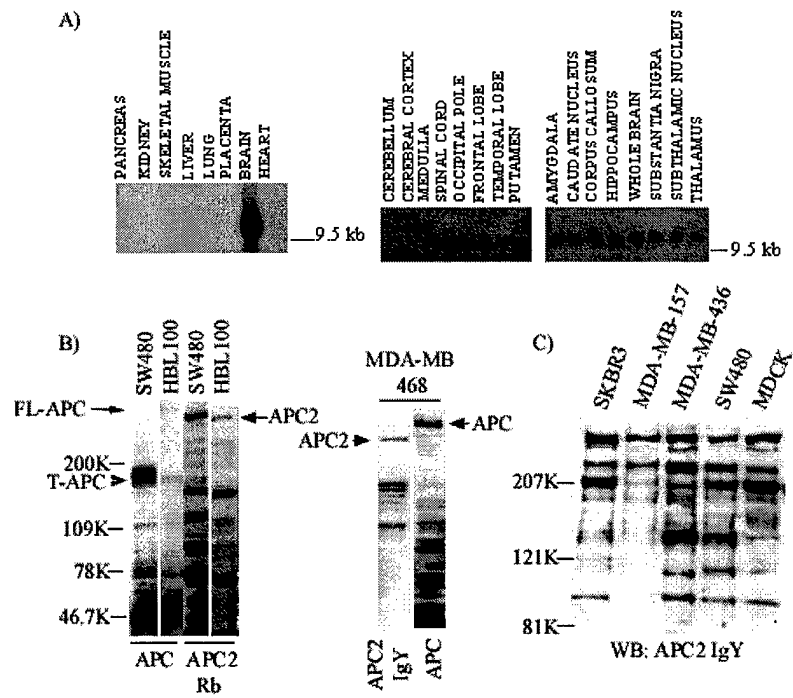


Figure 3

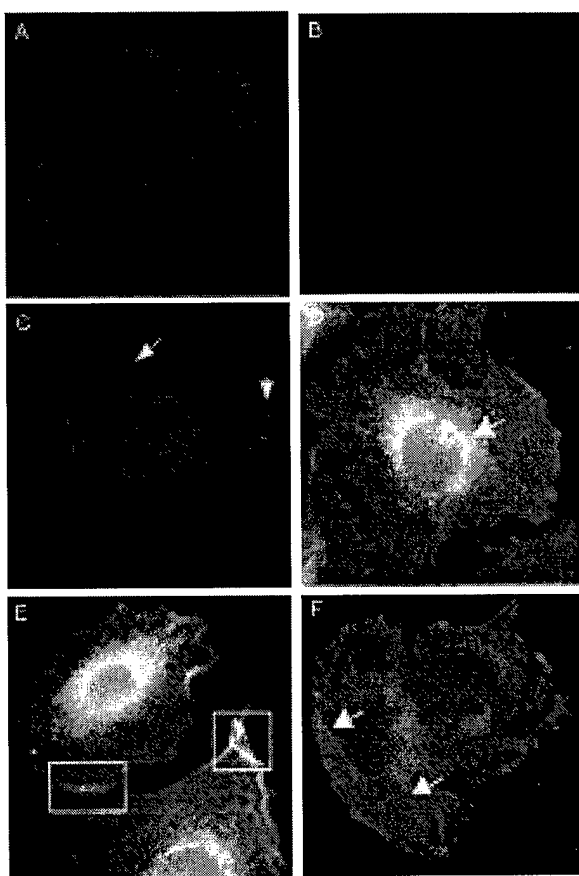


Figure 4

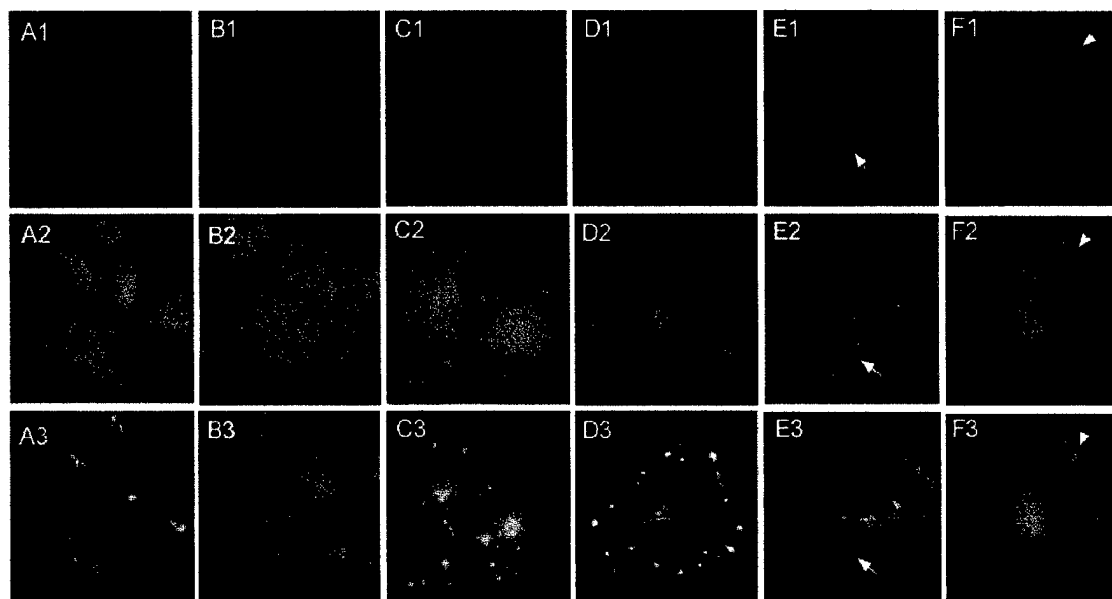


Figure 5

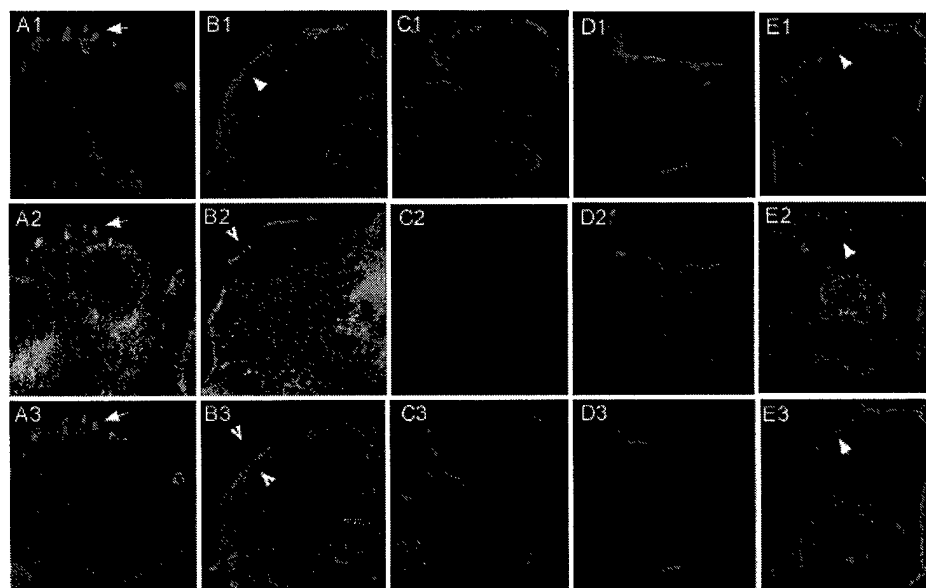


Figure 6

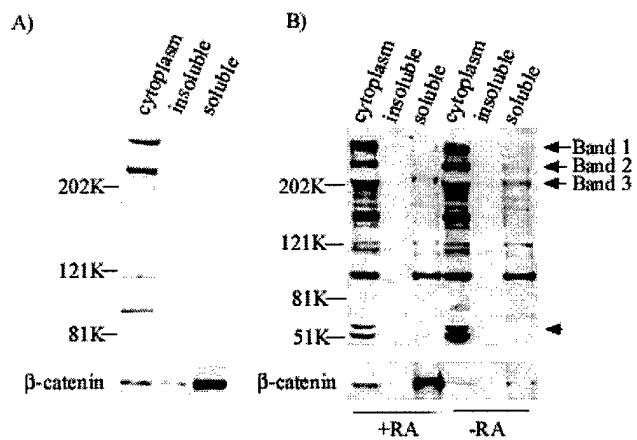


Figure 7

